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Coagulase-negative *Staphylococcus* species in bulk milk: Prevalence, distribution, and associated subgroup- and species-specific risk factors

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ABSTRACT

Coagulase-negative staphylococci (CNS) have become the main pathogens causing bovine mastitis in recent years. A huge variation in species distribution among herds has been observed in several studies, emphasizing the need to identify subgroup- and species-specific herd-level factors to improve our understanding of the differences in ecological and epidemiological nature between species. The use of bulk milk samples enables the inclusion of a large(r) number of herds needed to identify herd-level risk factors and increases the likelihood of recovering enough isolates per species needed for conducting subgroup- and, eventually, species-specific analyses at the same time. This study aimed to describe the prevalence and distribution of CNS species in bulk milk samples and to identify associated subgroup- and species-specific herd-level factors. Ninety percent of all bulk milk samples yielded CNS. *Staphylococcus equorum* was the predominant species, followed by *Staphylococcus haemolyticus* and *Staphylococcus epidermidis*. A seasonal effect was observed for several CNS species. Bulk milk samples from herds with a loose-pack or a tiestall housing system were more likely to yield CNS species compared with herds with a freestall barn, except for *S. epidermidis*, *Staphylococcus simulans*, and *Staphylococcus cohnii*. In September, herds in which udders were clipped had lower odds of yielding *Staphylococcus chromogenes*, *S. simulans*, and *Staphylococcus xylosus*, the CNS species assumed to be most relevant for udder health, in their bulk milk than herds in which udder clipping was not practiced. Bulk milk of herds participating in a monthly veterinary udder health-monitoring program was more likely to yield these 3 CNS species. Herds always receiving their milk quality premium or predisinfecting teats before attachment of the milking cluster had lower odds of having *S. equorum* in their bulk milk. Herds not using a single dry cotton or paper towel for each cow during premilking udder preparation were more likely to have *S. cohnii*-positive bulk milk. Herds in which flushing with hot water or steam of the milking cluster after having milked a cow with a (sub)clinical mastitis was applied, were less likely to yield *S. simulans*, *S. haemolyticus*, and *S. cohnii* in their bulk milk. Always wearing gloves during milking decreased the odds of having *Staphylococcus devriesei*-positive bulk milk. Tap water from the public drinking system used as drinking water increased the odds of yielding *S. simulans* in the bulk milk. In conclusion, CNS are highly prevalent in bulk milk and might originate from the environment for some species (we hypothesize this is true for *S. equorum* or *S. cohnii*), or from within the udder (e.g., for *S. simulans*). Studies collecting bulk milk and quarter milk samples at the same time along with environmental samples are needed to determine the exact origin of the different (subgroups of) CNS species present in bulk milk using strain-typing techniques.
Key words: dairy cattle, risk factor, coagulase-negative staphylococci, herd

INTRODUCTION

Coagulase-negative staphylococci have become the main bovine subclinical mastitis pathogens in several regions and countries (Piepers et al., 2007; Reyher et al., 2011; Sztachańska et al., 2016). Research relying on genotypic identification demonstrated the abundant presence of diverse CNS species in different bovine habitats, such as the cows' environment (Piessens et al., 2011), milk samples (Santos et al., 2008; Park et al., 2011; Persson Waller et al., 2011), and udder-related habitats (Taponen et al., 2008; De Visscher et al., 2014, 2016a). Describing CNS prevalence and distribution in those habitats and identifying associated risk factors, as has been done for other mastitis pathogens, is the obvious next step toward a better understanding of the variation in epidemiological and ecological nature

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among species (Zadoks et al., 2001; Østerås et al., 2006; Fox, 2009). However, species-specific research requires extensive studies including a considerable number of herds, cows, and quarters to obtain enough isolates of each (subgroup of) species for further analyses (Vanderhaeghen et al., 2015) and to identify associated factors.

Bulk milk is a convenient matrix, as it is readily available as part of the (regulatory) milk quality screening programs in different countries and includes milk of all lactating animals in the herd whose milk is sold (and not discarded). Bulk milk has already shown its value for the identification of herd-level management practices associated with the presence of *Staphylococcus aureus* (Olde Riekerink et al., 2010). Using bulk milk rather than composite or quarter milk samples conveniently enables the inclusion of a large(r) number of herds needed specifically to study herd-level risk factors. Bulk milk has actually been used for detecting genes encoding CNS virulence factors (Bertelloni et al., 2015) and for evaluating the suitability of mannitol salt agar for CNS recovery (De Visscher et al., 2013). The current study aimed (1) to describe the herd-level prevalence and distribution of CNS species in Flemish dairy herds using bulk milk samples, (2) to assess the variation in the presence of (subgroups of) different CNS species among herds, and (3) to identify associated herd-level risk factors.

MATERIALS AND METHODS

Herds, Samples, and Data

In the provinces of Antwerp, Flemish Brabant, Limburg, East Flanders, and West Flanders, 19, 5, 11, 28, and 37 herds (n = 100) were randomly selected from the database of the Flanders Milk Control Center (MCC Flanders, Lier, Belgium) using the RAND function (Excel 2010, Microsoft Corp., Redmond, WA) matching the relative distribution of dairy herds per province in Flanders. The selected herds had an average milk quota of 449,000 kg/yr, ranging between 92,000 and 1,500,000 kg. A herringbone milking parlor was the most commonly found milking parlor setup (n = 52), followed by a tiestall (n = 20), a tandem parlor (n = 18), a side-by-side parlor (n = 6), an automated milking system (n = 2), and a rotary parlor (n = 1). In one herd, a herringbone milking parlor was replaced by an automated milking system during the year of sampling (i.e., 2013). Fifty herds only housed dairy cattle, whereas the other half also farmed pigs, beef cattle, or poultry.

Bulk milk samples were collected 3 times per herd with a 3-mo interval in 2013 (March, June, and September) as part of the mandatory milk quality screening program executed by MCC and used for this study. Bulk milk-quality data were retrieved from MCC. The geometric mean bulk milk SCC in the month of sampling (March, June, and September) was calculated based on 4 weekly records and revealed a minimum, a maximum, and an arithmetic average of 74,000, 539,000, and 235,000 cells/mL, respectively. Several herd-level factors potentially associated with the presence of (subgroups of) CNS species in bulk milk were collected through a questionnaire and covered the entire study period (i.e., January–December 2013; Table 1).

Laboratory Analyses

Bulk milk samples (n = 300) were collected at the dairy farm and plated on mannitol salt agar (**MSA**; Oxoid, Erembodegem, Aalst, Belgium; 1 bulk milk sample per plate) in the laboratory of MCC Flanders (De Visscher et al., 2013). After an aerobic incubation of 24 h, plates were transported at 4°C to the Mastitis and Milk Quality Research Lab (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium). All phenotypically different colony types were counted, picked up (1 colony per colony type), and subcultured on esculin blood agar (Oxoid) to obtain pure cultures. Afterward, plates were aerobically incubated (37°C) for another 24 h and again examined. All recovered isolates suspected of belonging to the group of CNS were stored at −80°C or immediately subjected to species identification using transfer RNA intergenic spacer PCR (**tDNA-PCR**). If no identification could be obtained with this technique, 16S rRNA gene sequencing was used (Supré et al., 2009).

CNS Species Distribution

The CNS species distribution in all bulk milk samples (n = 300) in 100 Flemish dairy herds at the 3 sampling occasions (i.e., March, June, and September 2013) was computed (Table 2).

Questionnaire Data and Statistical Analysis

Before any statistical analysis was performed, observations were checked for unlikely or missing values. Complete questionnaire data were available from 95 herds.

Multilevel logistic regression models were fit applying reweighted iterative generalized least squares and first-order penalized quasi-likelihood in MLwiN 2.16 (Centre for Multilevel Modeling, University of Bristol, Bristol, UK). Month of sampling (March, June, and September) was forced into all models as a fixed effect and herd was added as a random effect to account for